Possible Errors in Assay for β-Glycosidase Activity

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Cecal homogenates were assayed for the enzymes β-glucosidase, β-glucuronidase, and β-galactosidase. Anaerobic incubation with the addition of excess 3,4-dichloronitrobenzene, a substrate for nitroreductase, significantly increased the detection of the β-glycosidase enzymes' activities.

The major microbial β-glycosidases in the intestinal tract and its contents are β-glucuronidase (BGr), β-glucosidase (BGl), and β-galactosidase (BGa) (7). These glycosidases are important in toxicology because they release noxious metabolites from nontoxic glycosides and thus prolong the lifetime of these toxicants in the body. For example, the mutagenic metabolites 6-hydroxy-1-acetamidopyrene (10, 14, 16), quercetin (2, 12, 18), and kaempferol (1) are released by the hydrolytic actions of BGr, BGl, and BGa on their respective glycosides. In addition, there is evidence for the presence of mucosal BGr in the intestinal tract (2, 19, 20) and some proof for the existence of mucosal BGa (17). However, it has been suggested that mucosal BGl exists only in the infant rat and disappears by the third week of life (9). Because changes in the activity of these enzymes alter the toxicity of many environmental chemicals, accurate analysis is important and errors could lead to inexact or possibly incorrect conclusions.

Glycosides of *p*-nitrophenol are frequently employed as substrates in the analysis of β-glycosidase activities, because their enzymatic hydrolysis releases *p*-nitrophenol, which is readily detected by spectroscopic analysis at 405 nm. A review of studies in which glycosides of *p*-nitrophenol are employed to determine β-glycosidase activity indicates that most investigators do not anticipate the confounding effect of mucosal or microbial nitroreductase on the determination of β-glycosidase activity (5). Nevertheless, there was one attempt to reduce the biodegradation of *p*-nitrophenol by employing aerobic incubation (15). However, anaerobic conditions are necessary to support some of the intestinal microorganisms with β-glycosidase activity. As a result, previous estimates of β-glycosidase activities in studies employing the glycosides of *p*-nitrophenol as substrates may have been low. To ensure that differences in the analytical procedures do not influence the results obtained, both spectroscopic and gas-liquid chromatographic (GLC) analyses are employed in the current investigation of intestinal β-glycosidase activity.

This study was designed to investigate the limitations of using glycosides of *p*-nitrophenol as substrates to determine the activities of BGr, BGl, and BGa. The objectives of the reported experiments were (i) to determine whether intestinal nitroreductase activity affected the analytical estimation of β-glycosidase activities when using the glycosides of *p*-nitro-phenol as substrates, (ii) to determine whether there are differences in the activities of the glycosidase enzymes when the samples are incubated under either anaerobic or aerobic conditions, (iii) to investigate whether or not BGr, BGl, and BGa are uniformly affected by the presence of intestinal nitroreductase and incubation under aerobic conditions, and (iv) to compare the estimation of β-glycosidase activities by GLC and spectroscopic analyses.

In the initial study, which determined the recovery of *p*-nitrophenol from cecal homogenates, six 21-day-old male Fischer 344 rats were used (Table 1). Adult rats (weight, 319 to 351 g) were employed to determine β-glycosidase activities (Table 2; Fig. 1). Animals had free access to 5001 Purina lab chow and water. The rats were sacrificed by CO_2 asphyxiation and lateral pneumothorax. The cecum from each animal ($n = 6$) was excised at autopsy and homogenized under CO_2 in 20 ml of prereduced sterile buffer (0.2 to 0.3 g of tissue per ml) (11). Aliquots (1.5 ml) of the six homogenates were transferred to individual sterile 30-ml serum bottles containing 6 ml of sterile prereduced buffer in an anaerobic chamber with an atmosphere of 85% N_2, 5% CO_2, and 10% H_2 (Coy Laboratory Products Inc., Ann Arbor, Mich.). The chamber atmosphere was monitored with an oxygen-hydrogen monitor (Coy Laboratory Products Inc.). Six aliquots of each cecal homogenate were dispensed anaerobically so that the BGr, BGl, and BGa activities of each animal could be determined anaerobically in the presence or absence of 3,4-dichloronitrobenzene (DCNB). The bottles were capped with sterile rubber stoppers, removed from the chamber, and placed on ice. A duplicate set of six samples was prepared under aerobic conditions and also was placed on ice. To half of the anaerobic and aerobic samples, 37.5 μl of a dimethyl sulfoxide (DMSO) solution containing 0.17 g of DCNB per ml and 0.13 g of either *p*-nitrophenyl-β-D-glucuronide (pNPGlr) *p*-nitrophenyl-β-D-galactopyranoside (pNPGal), or *p*-nitrophenyl-β-D-glucopyranoside (pNPGlu) (Sigma Chemical Company, St. Louis, Mo.) per ml was injected through the rubber stoppers into the bottles. To the remaining samples, 37.5 μl of a DMSO solution containing only 0.13 g of either pNPGlr, pNPGlu, or pNPGal per ml was injected. Corresponding cecal controls (6 ml of buffer [11], 1.5 ml of cecal homogenate, 37.5 μl of DMSO), a reagent control (7.5 ml of buffer, 37.5 μl of DMSO), and a substrate control (7.5 ml of buffer, 37.5 μl of 0.13-g/ml *p*-NPGlr, *p*-NPpGlu, or *p*-NPGal) also were prepared for incubation. The bottles were then shaken slowly in a Model G-76 Gyrotory water bath shaker (New Brunswick Scientific Inc., Edison, N.J.) for 1 h at 37°C and were placed on ice.

For spectroscopic analysis, a 2.5-ml aliquot of each sample was diluted 1:50 and filtered through a filter (pore size, 0.15

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TABLE 1. Recovery of p-nitrophenol from incubated
tissue homogenates[a]

Assay mix	% p-NP recovered (mean ± SEM)	
	Small intestine	Large intestine
p-NP + homogenate	55.1 ± 2.1	65.3 ± 2.1
p-NP + boiled homogenate	70.1 ± 7.6	84.8 ± 2.1
p-NP + DCNB + homogenate	92.7 ± 1.9	97.0 ± 0.6

[a] p-Nitrophenol (p-NP) was recovered from tissue samples (n = 6) incubated at 37°C for 1 h with 125 μg of p-NP. Data were determined by the GLC method described in the text. Rats were 21 days old.

FIG. 1. β-Glycosidase activities in rat cecal homogenates as measured by spectroscopic and GLC analyses. The effect of adding excess DCNB was studied. An asterisk denotes a significant difference (P < 0.05) between open and closed bars.

μm). The filtrate was read against its corresponding cecal control at an optical density at 405 nm on a Stasar II spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). The concentration of released p-nitrophenol was extrapolated from a p-nitrophenol standard curve.

For GLC analysis, the remainder of the incubated samples was transferred to 25-ml screw-capped tubes containing 3 g of NaHCO$_3$ and placed on ice. The samples were acetylated by the addition of 0.5 ml of acetic anhydride as described by Coutts et al. (6). After the bubbling stopped, the samples were extracted three times with separate 5-ml volumes of toluene. Extracts were analyzed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a ^{63}Ni electron capture detector, Hewlett-Packard 7673 autosampler, and a coiled glass column (internal diameter, 1.8 m by 4 mm), packed with 1.5% OV-17 plus 1.95% QF-1 on 80/100-mesh Gas Chrom Q column packing (Applied Science Laboratories Inc., State College, Pa.). Operating conditions were 185°C, 330°C, and 160°C for the injector, detector, and oven, respectively. The N$_2$ carrier gas was regulated at 50 cm^3/min.

Previous unpublished data indicated poor recovery of p-nitrophenol after incubation for 1 h at 37°C with cecal homogenates (Table 1). Boiling the homogenate before use or adding DCNB, a substrate for nitroreductase, significantly increased the recovery of p-nitrophenol. The reduction in p-nitrophenol recovery in the spiked boiled tissue samples may be due to heat alteration of the substrate to an undetectable or extractable metabolite (Table 1). Because heat inactivation is not an instantaneous process, there may be some p-nitrophenol reduction to p-aminophenol prior to total heat inactivation. To determine the influence of tissue and microbial nitroreductase enzymes on the analysis of BGr, BGl, and BGa activities, cecal homogenates were incubated anaerobically with the re-
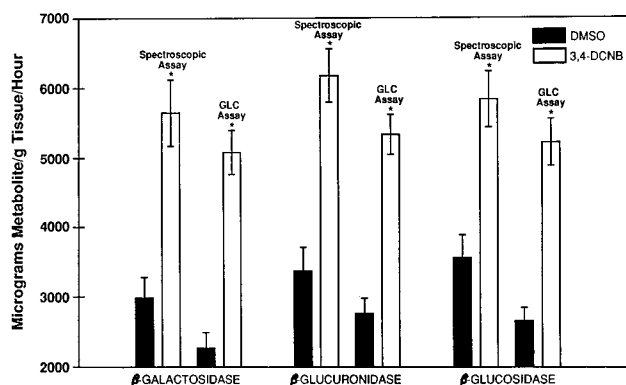
spective glycosides of p-nitrophenol in the presence and absence of DCNB, a previously employed substrate, for the determination of nitroreductase activity (3, 4). In the absence of DCNB, there was about a twofold decline in the recovery of p-nitrophenol and thus the estimated activity of all three enzymes (Fig. 1). Results from GLC and spectroscopic analyses were in good agreement. The greater specificity of the GLC analysis probably accounts for its lower response. However, differences between the GLC and spectroscopic analyses were not significant.

The activities of the three glycosidase enzymes were determined under either aerobic or anaerobic conditions and in the presence or absence of DCNB (Table 2). The maximal activity for all three enzymes was obtained under anaerobic conditions in the presence of excess DCNB. The addition of excess DCNB under anaerobic conditions resulted in β-glycosidase activities that were twice as great as those obtained from anaerobic incubation without DCNB and 1.5 times as large as those obtained from aerobic incubation without DCNB (Table 2). On the other hand, only the activity of BGr was significantly greater, under protected anaerobic incubation, when compared with that obtained under aerobic conditions in the presence of DCNB. Moreover, without DCNB, either there was no significant difference between the enzyme activities determined under aerobic and anaerobic conditions (BGr) or the activities were higher in the samples (BGl and BGa) incubated under aerobic conditions (Table 2).

TABLE 2. Effect of aerobic incubation and DCNB on glycosidase activity

Incubation conditions[a]	Activity (mean ± SEM)[b]		
	BGa	BGr	BGl
Anaerobic			
+DCNB	5.08 ± 0.32	5.33 ± 0.29	5.22 ± 0.34
−DCNB	2.27 ± 0.23	2.76 ± 0.22	2.66 ± 0.19
Aerobic			
+DCNB	4.67 ± 0.20	4.59 ± 0.16	4.77 ± 0.16
−DCNB	3.48 ± 0.29	3.29 ± 0.22	3.47 ± 0.20
Comparisons	All > Anr−; Anr+, Aer+ > Aer−	Anr+ > All; Aer+ > Aer−, Anr−	All > Anr−; Anr+, Aer+ > Aer−

[a] Duncan's multiple range test (8) and Student's t test (18) were used as aids in the interpretation of the data in this study. An F test was used to assess homogeneity of variance (18). Comparisons were considered significantly different at P < 0.05. Anr+, anaerobic incubation with DCNB; Anr−, anaerobic incubation without DCNB; Aer+, aerobic incubation with DCNB; Aer−, aerobic incubation without DCNB.

[b] Milligrams of p-nitrophenol per gram of tissue per hour. Data were determined by the GLC assay described in the text.

Nitroreductase(s) catalyzes the reduction of the hydrolysis product, *p*-nitrophenol, to *p*-aminophenol. Because *p*-aminophenol is not detected by either the GLC or the spectroscopic assay, this loss of product would counteract the greater rate of hydrolysis and, under anaerobic conditions, would compromise the recovery of *p*-nitrophenol and would therefore result in reduced estimations of β-glycosidase activities. On the other hand, under aerobic conditions, the reduction of *p*-nitrophenol, by nitroreductase, would be inhibited at the molecular level in the presence of oxygen (13). Adding excess DCNB protected *p*-nitrophenol and optimized the estimation of β-glycosidase activities (Table 1; Table 2). Similar results were obtained by both the GLC and the spectroscopic analyses (Fig. 1).

In summary, results from this study indicate that when employing the glycosides of *p*-nitrophenol as substrates to analyze β-glycosidase activity, the sensitivity of the assay to microbial and/or mucosal nitroreductase(s) and aerobic incubation conditions must be taken into account. For analysis of microbial or mucosal β-glycosidase activity, it is recommended that analysts avoid using the glycosides of *p*-nitrophenol and select an alternate substrate whose metabolite is not so susceptible to degradation by other enzymes. For example, the glycosides of 4-methylumbelliferyl or phenolphthalein are commercially available. If the glycosides of *p*-nitrophenol are employed, however, excess quantities of a competitive substrate for nitroreductase, such as DCNB, should be added to the incubation mixture to protect the released *p*-nitrophenol from reduction to the amine. In addition, the assay should be conducted under anaerobic conditions for optimum results.

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